

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Respectable Assistant Commissioner for Patents
Washington, D.C. 20231
Patent Application
S I R:

December 23, 1997

Transmitted herewith for filing are the specification and claims of the patent application of:

David Stern, Shi D. Yan and Benjamin Wolozin _____ for
Inventor(s)

A METHOD FOR EVALUATING THE ABILITY OF A COMPOUND TO INHIBIT NEUROTOXICITY

Title of Invention

Also enclosed are:

X 3 sheet(s) of _____informal X formal drawings.

_____ Oath or declaration of Applicant(s).

_____ A power of attorney

_____ An assignment of the invention to _____

_____ A Preliminary Amendment

X A verified statement to establish small entity status under 37 C.F.R. §1.9 and §1.27.

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CLAIMS AS FILED, LESS ANY CLAIMS CANCELLED BY AMENDMENT

	NUMBER FILED		NUMBER EXTRA*		RATE		FEE	
					SMALL ENTITY	OTHER ENTITY	SMALL ENTITY	OTHER ENTITY
Total Claims	33-20	=	13	X	\$11	\$22	=	\$143.00
Independent Claims	4 -3	=	1	X	\$41	\$82	=	\$41.00
Multiple Dependent Claims Presented: _____ Yes <u>X</u> No					\$135	\$270	=	\$0
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Applicants: David Stern et al.
U.S. Serial No.: Not yet known
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Letter of Transmittal
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- ☐ Please charge Deposit Account No. _____ in the amount of \$ _____.
- ☒ The Commissioner is hereby authorized to charge any additional fees which may be required in connection with the following or credit any over-payment to Account No. 03-3125:
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- ☒ Three copies of this sheet are enclosed.
- ☐ A certified copy of previously filed foreign application No. _____ filed in _____ on _____.
Applicant(s) hereby claim priority based upon this aforementioned foreign application under 35 U.S.C. \$119.
- ☒ Other (identify) One loose set of drawings and an Express Mail Certificate of Mailing bearing label No. EM 165 673 542 US.
- _____
- _____
- _____

Respectfully submitted,



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For : A METHOD FOR EVALUATING THE ABILITY OF A
COMPOUND TO INHIBIT NEUROTOXICITY

1185 Avenue of the Americas
New York, New York 10036
December 23, 1997

Assistant Commissioner for Patents
Washington, D.C. 20231

Box: Patent Application

Sir:

**EXPRESS MAIL
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the United States Postal Service "Express Mail Post Office to
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Serial or Patent No.: Not yet known Docket No: 54202/JPW/SBS
Filed or Issued: Herewith
Title of Invention or Patent: A Method For Evaluating The Ability Of A Compound
To Inhibit Neurotoxicity

VERIFIED STATEMENT (DECLARATION) CLAIMING
SMALL ENTITY STATUS UNDER 37 C.F.R. §1.9(f)
AND §1.27(d) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

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STATES OF AMERICA
NAME OF STATE: _____
CITATION OF STATUTE: _____
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§§501(a) and 501(c)(3) IF LOCATED IN THE UNITED STATES OF AMERICA
☐ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE
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NAME OF STATE: _____
CITATION OF STATUTE: _____

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 C.F.R. §1.9(e)* for purposes of paying reduced fees under 35 U.S.C. §41(a) and 41(b), with regard to the invention entitled A Method For Evaluating The Ability Of A Compound To Inhibit Neurotoxicity

by inventor(s) David Stern, Shi Du Yan and Benjamin Wolozin

described in:

☒ the specification filed herewith
application serial no. _____ filed _____
patent no. _____ issued _____

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive each individual, concern, or organization known to have rights to the invention is listed below^a and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 C.F.R. §1.9(d)* or a nonprofit organization under 37 C.F.R. 1.9(e)*

^aNOTE: Separate verified statements are required from each person, concern, or organization having rights to the invention averring to their status as small entities. 37 C.F.R. §1.27.

Name: N/A

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☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

Applicants: David Stern et al.
U.S. Serial No.: Not yet known
Filed: Herewith

Small Entity/Nonprofit
Page -2-

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. 37 C.F.R. §1.28(b)*.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing: Mr. Jack M. Granowitz
Title In Organization: Executive Director, Columbia Innovation Enterprise
Address: Amsterdam & 120th Street - Suite 363 New York, New York 10027

Signature: *Jack M. Granowitz*
Date Of Signature: December 4, 1998

**Application
for
United States Letters Patent**

To all whom it may concern:

*Be it known that we, David Stern, Shi D. Yan and Benjamin Wolozin
have invented certain new and useful improvements in
A METHOD FOR EVALUATING THE ABILITY OF A COMPOUND TO INHIBIT NEUROTOXICITY
of which the following is a full, clear and exact description.*

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"SECRET" 4946680

A Method For Evaluating The Ability Of A Compound To
Inhibit Neurotoxicity

5

The invention disclosed herein was made with Government support from National Institute of Aging/National Institutes of Health grants K01AG00690, R01AG14103, and R01HL56881. Accordingly, the U.S. Government has certain
10 rights to this invention.

Background of the Invention

Throughout this application, various publications are
15 referenced by author and date. Full citations for these publications may be found listed alphabetically at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference in order to more
20 fully describe the state of the art.

Neurologic disease represents a particularly problematic area for the identification of therapeutic compounds which are effective in humans. Alzheimer's disease (AD), for
25 example, has come under intense scrutiny, but no effective therapies have yet been identified. There is a critical need for an effective system by which therapeutic compounds can be identified.

30 Extracellular accumulations of amyloid in neuritic plaques composed predominately of amyloid-beta peptide (A β) are pathognomonic features of AD (Haas et al., 1994; Kosik, et al., 1994; Yankner, et al., 1996; Goedert, et al., 1993; Trojanowski, et al., 1994). These lesions increase in
35 number and volume over time resulting in an apparent replacement of the neuronal cell population (Haas et al., 1994; Kosik, et al., 1994; Yankner, et al., 1996; Goedert, et al., 1993; Trojanowski, et al., 1994; Cummings, et al., 1995.), and are closely associated with neuronal toxicity

leading to dementia.

In AD it is widely accepted that later in the course of the disease, when A β fibrils are abundant, nonspecific
5 interactions of such fibrils with the cell surface may be frequent and disruptive for cellular functions (Yankner, B., et al., 1990; Cotman, et al. 1995; Mattson, et al., 1995; Hensley, et al., 1994; Behl, et al., 1994; Younkin, et al., 1995). A β fibrils can disrupt plasma membranes,
10 causing changes in course of the disease, when A β fibrils are present at lower levels (and monomers/oligomers predominate, as opposed to fibrils), higher affinity interactions with cellular surfaces are more likely to be relevant. The immunoglobulin superfamily receptor RAGE
15 (Receptor for Advanced Glycation Endproduct), expressed by neurons and microglia, is present at high levels in AD brain, both in areas of affected brain parenchyma (at the antigen and mRNA levels) and in nearby vasculature. RAGE is a receptor with nanomolar affinity for A β
20 monomer/oligomer, as well as for fibrils (Yan, et al., 1996). In culture, cells expressing RAGE display enhanced susceptibility to A β -induced cellular dysfunction compared with those expressing lower levels of RAGE, or those in which the receptor is blocked. Consistent with a role for
25 A β -receptor interactions in early perturbation of neuronal functions, relevant outcomes of A β binding to neuronal RAGE include activation of nuclear factor-KB (NF-kB), induction of heme oxygenase type 1 and expression of macrophage-colony stimulating factor (M-CSF), each of
30 which can be demonstrated in AD brain (Yan, et al., 1996; Yan, et al. 1997).

Mutant presenilins 1 and 2 are closely associated with most cases of early onset familial AD (Haas, et al., 1996;
35 Dwji, et al., 1996; Tanzi, et al., 1996; Hardy, et al. 1997). Furthermore, presenilin-2 may be involved in cellular pathways which eventuate in programmed cell

death; a mutant form of presenilin-2 results in expression of a molecule causing increased basal apoptosis in nerve growth factor-differentiated PC12 cells (Wolozin, et al., 1996). PC12 cells, stably transfected with amyloid precursor protein (APP) show increased levels of apoptosis after serum withdrawal. Cellular stress increases synthesis of APP and, depending on the particular stress, secretion of either APP or $A\beta$. Enhanced activation of presenilin-2 protein expression might not only increase the tendency toward apoptosis, but by activating apoptotic signals, may trigger a stress response and increase production of $A\beta$, leading to neurodegeneration (Wolozin, et al., 1996).

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Summary of the Invention

- 5 The present invention provides a method for evaluating the ability of a compound to inhibit neurotoxicity which comprises (a) contacting a cell which expresses a receptor for advanced glycation end product protein and a mutant presenilin-2 protein in a cell culture with the compound;
- 10 (b) determining the level of cell death in the cell culture; and (c) comparing the level of cell death determined in step (b) with the amount determined in the absence of the compound so as to evaluate the ability of the compound to inhibit neurotoxicity.
- 15 Additionally, the present invention provides a method for evaluating the ability of a compound to inhibit binding of an amyloid- β peptide to a receptor for advanced glycation end product which comprises (a) contacting a cell which
- 20 expresses a mutant presenilin-2 protein and a receptor for advanced glycation end product protein with amyloid- β protein and the compound; (b) determining the amount of amyloid- β peptide bound to the cell; (c) comparing the amount of bound amyloid- β peptide determined in step (b)
- 25 with the amount determined in the absence of the compound so as to evaluate the ability of the compound to inhibit binding of the amyloid- β peptide to the receptor for advanced glycation end product.
- 30 The present invention also provides a pharmaceutical composition which comprises a compound capable of inhibiting neurotoxicity and a pharmaceutically acceptable carrier.
- 35 Moreover, the present invention additionally provides a method for treating a neurodegenerative condition in a subject which comprises administering to the subject an

amount of a pharmaceutical composition, effective to treat the neurodegenerative condition in the subject.

Further, the present invention also provides a transgenic
5 non-human animal whose somatic and germ cells contain and
express a gene encoding mutant human presenilin-2 protein
and whose somatic and germ cells contain and express a
gene encoding human receptor for advanced glycation end
10 product protein, the genes having been introduced into the
animal or an ancestor of the animal at an embryonic stage
and wherein the gene may be operably linked to an
inducible promoter element.

The invention further provides a method for identifying
15 whether a compound is capable of ameliorating a
neurodegenerative condition in an animal comprising (a)
administering the compound to a transgenic animal, wherein
the animal exhibits a neurodegenerative condition; (b)
measuring the level of neurodegeneration in the animal;
20 and (c) comparing the level of neurodegeneration measured
in step (b) with the level of neurodegeneration measured
in the animal in the absence of the compound so as to
identify whether the compound is capable of ameliorating
the neurodegenerative condition in the animal.

25 Finally, the invention provides a cell comprising a
recombinant nucleic acid encoding mutant presenilin-2
protein and encoding receptor for advanced glycation
endproduct protein.

Brief Description of the Figures

- 5 **Figures 1A-1B.** Immunostaining of stably RAGE-transfected (A) or mock-transfected (B) PC12 cells. These experiments employed anti-human RAGE IgG and methods described in Yan et al., 1996.
- 10 **Figure 2.** Immunoblotting was performed on extracts of stably RAGE-transfected (lane 2) or mock-transfected (lane 1) PC12 cells. These experiments employed anti-human RAGE IgG and methods described in Yan et al., 1996.
- 15 **Figure 3.** Stably RAGE-transfected PC12 cells or mock-transfected controls were co-transfected with presenilin 2 (RAGE/PS2) and exposed to the indicated concentration of A β , as indicated. Apoptosis was determined 18 hours later by TUNEL staining.

Detailed Description of the Invention

5 This invention provides a method for evaluating the ability of a compound to inhibit neurotoxicity which comprises (a) contacting a cell which expresses a receptor for advanced glycation end product protein and a mutant presenilin-2 protein in a cell culture with the compound;
10 (b) determining the level of cell death in the cell culture; and (c) comparing the level of cell death determined in step (b) with the amount determined in the absence of the compound so as to evaluate the ability of the compound to inhibit neurotoxicity.

15 This invention also provides a method for evaluating the ability of a compound to inhibit cytotoxicity in a non-neuronal cell which comprises (a) contacting a cell which expresses a receptor for advanced glycation end product
20 protein and a mutant presenilin-2 protein in a cell culture with the compound (b) determining the level of cell death in the cell culture; and (c) comparing the level of cell death determined in step (b) with the amount determined in the absence of the compound so as to
25 evaluate the ability of the compound to inhibit cytotoxicity.

In this embodiment, the cell may be contacted with the compound and amyloid- β peptide. The cell may be contacted
30 with amyloid- β peptide simultaneously or the cell may be contacted with amyloid- β peptide and the compound at different times. The compound may be capable of specifically binding to amyloid- β peptide. The compound may bind to amyloid- β peptide at the site where the
35 receptor for advanced glycation end product interacts. The compound may be a soluble extracellular portion of a receptor for advanced glycation end product. The compound

may be bound to a solid support. The compound may be expressed on the surface of a cell. The compound may be present on the surface of a cell.

5 In the present invention the cell may be a neuronal cell, an endothelial cell, a glial cell, a microglial cell, an astrocyte, a smooth muscle cell, a somatic cell, a bone marrow cell, a liver cell, an intestinal cell, a germ cell, a myocyte, a mononuclear cell, a mononuclear
10 phagocyte, a tumor cell, a stem cell, or a PC12 cell. The cell may be under oxidant stress. The compound may be a peptide, a peptidomimetic, a nucleic acid, a polymer, or a small molecule. The compound may be bound to a solid support. The presenilin-2 may be a mutant or non-mutant
15 form of presenilin-2. The mutant form of presenilin-2 may be in the form of a deletion, substitution, insertion, or point mutation. The presenilin-2 may be of human or non-human origin. The mutant presenilin-2 protein may be overexpressed. The receptor for advanced glycation end
20 product may be overexpressed.

One embodiment of this invention is a method for evaluating the ability of a compound to inhibit binding of an amyloid- β peptide to a receptor for advanced glycation
25 end product which comprises (a) contacting a cell which expresses a mutant presenilin-2 protein and a receptor for advanced glycation end product protein with amyloid- β protein and the compound; (b) determining the amount of amyloid- β peptide bound to the cell; (c) comparing the
30 amount of bound amyloid- β peptide determined in step (b) with the amount determined in the absence of the compound so as to evaluate the ability of the compound to inhibit binding of the amyloid- β peptide to the receptor for advanced glycation end product.

35

In this embodiment, the cell may be contacted with the compound and the amyloid- β peptide simultaneously or the

cell may be contacted with the amyloid- β peptide and the compound at different times. The compound may be capable of specifically binding to amyloid- β peptide. The compound may bind to amyloid- β peptide at the site where the receptor for advanced glycation end product interacts. The compound may be a soluble extracellular portion of a receptor for advanced glycation end product. The compound may be bound to a solid support. The compound may be expressed on the surface of a cell. The compound may be present on the surface of a cell.

In this embodiment, the cell may be a neuronal cell, an endothelial cell, a glial cell, a microglial cell, an astrocyte, a smooth muscle cell, a somatic cell, a bone marrow cell, a liver cell, an intestinal cell, a germ cell, a myocyte, a mononuclear cell, a mononuclear phagocyte, a tumor cell, a stem cell, or a PC12 cell. The compound may be a peptide, a peptidomimetic, a nucleic acid, a polymer, or a small molecule. The compound may be bound to a solid support. The presenilin-2 may be a mutant or non-mutant form of presenilin-2. The mutant form of presenilin-2 may be in the form of a deletion, substitution, insertion, or point mutation. The presenilin-2 may be of human or non-human origin. The mutant presenilin-2 protein may be overexpressed. The receptor for advanced glycation end product may be overexpressed.

Another embodiment of the present invention provides for a pharmaceutical composition which comprises a compound capable of inhibiting neurotoxicity, and a pharmaceutically acceptable carrier. The carrier may be a diluent, an aerosol, a topical carrier, an aqueous solution, a nonaqueous solution or a solid carrier.

35

Another embodiment of this invention provides for a method for treating a neurodegenerative condition in a subject

which comprises administering to the subject an amount of the provided pharmaceutical composition, effective to treat the neurodegenerative condition in the subject. The subject may be a mammal or a human. The administration in this embodiment may be intralesional, intraperitoneal, intramuscular, or intravenous injection; infusion; liposome mediated delivery; topical, nasal, oral, anal, ocular or otic delivery.

10 In this embodiment, the neurodegenerative condition may be associated with Alzheimer's disease, diabetes, senility, renal failure, hyperlipidemic atherosclerosis, neuronal cytotoxicity, Down's syndrome, dementia associated with head trauma, amyotrophic lateral sclerosis, myasthenia gravis, multiple sclerosis or neuronal degeneration. The neurodegenerative condition may be associated with spongiform encephalopathic disease, including but not limited to Creutzfeldt-Jakob Disease, Fatal Familial Insomnia, kuru, Gerstmann-Straussler-Scheinker Disease, bovine spongiform encephalopathy, feline spongiform encephalopathy, transmissible mink encephalopathy, zoological spongiform encephalopathy, Alper's Disease or scrapie. The neurodegenerative condition may be associated with degeneration of a neuronal cell in the subject. The neurodegenerative condition may be associated with the formation of an amyloid- β peptide fibril. The neurodegenerative condition may be associated with aggregation of amyloid- β peptide. The neurodegenerative condition may be due to oxidant or cellular stress. The neurodegenerative condition may be associated with infiltration of a microglial cell into a senile plaque. The neurodegenerative condition may be associated with activation of a microglial cell by an amyloid- β peptide.

35 Still another embodiment of the present invention provides for a transgenic non-human animal whose somatic and germ

cells contain and express a gene encoding mutant human presenilin-2 protein and whose somatic and germ cells contain and express a gene encoding human receptor for advanced glycation end product protein, the genes having
5 been introduced into the animal or an ancestor of the animal at an embryonic stage and wherein the gene may be operably linked to an inducible promoter element. The transgenic animal may be a mouse or other non-human mammal. The mutant presenilin-2 protein may be
10 overexpressed. The receptor for advanced glycation end product may be overexpressed. The presenilin-2 may be a mutant or non-mutant form of presenilin-2. The mutant form of presenilin-2 may be in the form of a deletion, substitution, insertion, or point mutation. The
15 presenilin-2 may be of human or non-human origin.

Yet another embodiment of the present invention provides for a screening method to identify drugs, compounds or agents capable of treating symptoms associated with
20 neurotoxicity wherein the mechanism of cell death is modeled by the cell culture or the transgenic animal. The screening method may be performed on a library of drugs, compounds or agents. The screening method may involved mass screening, large throughput, automated or robotic
25 processing and analysis, or combinations thereof.

In one embodiment of this invention, the screening method is provided as part of a screening kit. A screening kit may include a cell which expresses a receptor for advanced
30 glycation end product protein and a mutant or non-mutant presenilin protein. Additionally, a screening kit may include a cell which does not express receptor for advanced glycation end product protein or a presenilin protein. The kit may include a cell which expresses
35 receptor for advanced glycation end product protein but not presenilin protein or a cell which expresses a presenilin protein but not a receptor for advanced

glycation end product protein. A kit may also include buffers and reagents for the detection and measurement of cell death, cell lysis, cell viability, apoptosis, or other cellular functions. Additionally a kit may include
5 a solid support matrix.

An additional embodiment of the present invention provides a cell isolated from the transgenic animal which expresses a transgene encoding mutant presenilin-2 protein and a
10 transgene encoding a receptor for advanced glycation end product protein. The isolated cell may be a neuronal cell, a glial cell, a microglial cell, an astrocyte, an endothelial cell, a mononuclear cell, a tumor cell, a muscle cell, a bone marrow cell, a liver cell, an
15 intestinal cell, a germ cell, a myocyte, a mononuclear phagocyte, or a stem cell.

Another embodiment of the present invention provides for a method for identifying whether a compound is capable of
20 ameliorating a neurodegenerative condition in an animal comprising (a) contacting a cell isolated from a transgenic animal which expresses a transgene encoding mutant presenilin-2 protein and a transgene encoding a receptor for advanced glycation end product protein with
25 amyloid- β protein and the compound; (b) determining the amount of amyloid- β peptide bound to the cell; (c) comparing the amount of bound amyloid- β peptide determined in step (b) with the amount determined in the absence of the compound so as to evaluate the ability of the compound
30 to inhibit binding of the amyloid- β peptide to the receptor for advanced glycation end product.

Another embodiment of the present invention provides for a method for identifying whether a compound is capable of
35 ameliorating a neurodegenerative condition in an animal comprising (a) contacting a cell isolated from a transgenic animal which expresses a gene encoding mutant

presenilin-2 protein and which expresses a gene encoding a receptor for advanced glycation end product protein with the compound; (b) determining the level of cell death; (c) comparing the level of cell death determined in step (b) with the level determined in the absence of the compound so as to evaluate the ability of the compound to inhibit neurotoxicity.

Another embodiment of the present invention provides for a method for identifying whether a compound is capable of ameliorating a neurodegenerative condition in an animal comprising (a) administering the compound to the provided transgenic animal, wherein the animal exhibits a neurodegenerative condition; (b) measuring the level of neurodegeneration in the animal; and (c) comparing the level of neurodegeneration measured in step (b) with the level of neurodegeneration measured in the animal in the absence of the compound so as to identify whether the compound is capable of ameliorating the neurodegenerative condition in the animal. The administration in this embodiment may be intralesional, intraperitoneal, intramuscular, or intravenous injection; infusion; liposome mediated delivery; topical, nasal, oral, anal, ocular or otic delivery.

In this embodiment, the neurodegenerative condition may be associated with Alzheimer's disease, diabetes, senility, renal failure, hyperlipidemic atherosclerosis, neuronal cytotoxicity, Down's syndrome, dementia associated with head trauma, amyotrophic lateral sclerosis, myasthenia gravis, multiple sclerosis or neuronal degeneration. The neurodegenerative condition may be associated with spongiform encephalopathic disease, including but not limited to Creutzfeldt-Jakob Disease, Fatal Familial Insomnia, kuru, Gerstmann-Straussler-Scheinker Disease, bovine spongiform encephalopathy, feline spongiform encephalopathy, transmissible mink encephalopathy,

zoological spongiform encephalopathy, Alper's Disease or scrapie. The neurodegenerative condition may be associated with degeneration of a neuronal cell in the subject. The neurodegenerative condition may be associated with the formation of an amyloid- β peptide fibril. The neurodegenerative condition may be associated with aggregation of amyloid- β peptide. The neurodegeneration may be due to oxidant or cellular stress. The neurodegenerative condition may be associated with infiltration of a microglial cell into a senile plaque. The neurodegenerative condition may be associated with activation of a microglial cell by an amyloid- β peptide.

As used herein, the term "oxidant stress" encompasses the perturbation of the ability of a cell to ameliorate the toxic effects of oxidants. Oxidants may include hydrogen peroxide or oxygen radicals that are capable of reacting with bases in the cell including DNA. A cell under oxidant stress may undergo biochemical, metabolic, physiological and/or chemical modifications to counter the introduction of such oxidants. Such modifications may include lipid peroxidation, NF-kB activation, heme oxygenase type I induction and DNA mutagenesis. Also, antioxidants such as glutathione are capable of lowering the effects of oxidants. "Cellular stress" may also be induced by serum starvation or by the withdrawal or deprivation of other trophic factors which may perturb normal cellular function. Such perturbations may be by the same or by different mechanisms as that induced by oxidant stress.

As used herein, apoptotic cell death is programmed or gene-directed cell death. A hallmark of apoptosis is the activation of endonuclease that attacks cellular genomic DNA at the linker regions that connect nucleosomal units. Degradation of DNA ensues, producing DNA fragments that

can be observed as a distinct DNA ladder pattern.

As used herein, the term "neurotoxicity" encompasses the negative metabolic, biochemical and physiological effects
5 on a neuronal cell which may result in a debilitation of the neuronal cellular functions, including but not limited to neuronal cell death. Such functions may include memory, learning, perception, neuronal electrophysiology (ie. action potentials, polarizations and synapses),
10 synapse formation, both chemical and electrical, channel functions, neurotransmitter release and detection and neuromotor functions. Neurotoxicity may include neuronal cytotoxicity or neuronal cell death.

15 As used herein, the term "neuronal degeneration" encompasses a decline in normal functioning of a neuronal cell. Such a decline may include a decline in memory, learning, perception, neuronal electrophysiology (ie. action potentials, polarizations and synapses), synapse
20 formation, both chemical and electrical, channel functions, neurotransmitter release and detection and neuromotor functions. In the present invention, the subject may be a mammal or a human subject.

25 As used herein, the term "cytotoxicity" encompasses the negative metabolic, biochemical and physiological effects on a cell which may result in a debilitation of the cellular functions, including but not limited to cell death.

30

In the practice of any of the methods of the invention or preparation of any of the pharmaceutical compositions an "therapeutically effective amount" is an amount which is capable of inhibiting the binding of an amyloid- β peptide
35 with a receptor for advanced glycation eudproduct. Accordingly, the effective amount will vary with the subject being treated, as well as the condition to be

treated. For the purposes of this invention, the methods of administration are to include, but are not limited to, administration cutaneously, subcutaneously, intravenously, parenterally, orally, topically, or by aerosol.

5

As used herein, the term "suitable pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutically accepted carriers, such as phosphate buffered saline solution, water, emulsions such as an oil/water emulsion or a triglyceride emulsion, various types of wetting agents, tablets, coated tablets and capsules. An example of an acceptable triglyceride emulsion useful in intravenous and intraperitoneal administration of the compounds is the triglyceride emulsion commercially known as Intralipid®.

Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients.

This invention also provides for pharmaceutical compositions capable of inhibiting neurotoxicity together with suitable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions are liquids or lyophilized or otherwise dried formulations and include diluents of various buffer content (e.g., Tris-HCl., acetate, phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts), solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the

compound, complexation with metal ions, or incorporation of the compound into or onto particulate preparations of polymeric compounds such as polylactic acid, polglycolic acid, hydrogels, etc, or onto liposomes, micro emulsions, micelles, unilamellar or multi lamellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the compound or composition.

10

Controlled or sustained release compositions include formulation in lipophilic depots (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g., poloxamers or poloxamines) and the compound coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal and oral.

When administered, compounds are often cleared rapidly from the circulation and may therefore elicit relatively short-lived pharmacological activity. Consequently, frequent injections of relatively large doses of bioactive compounds may be required to sustain therapeutic efficacy. Compounds modified by the covalent attachment of water-soluble polymers such as polyethylene glycol, copolymers of polyethylene glycol and polypropylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinylpyrrolidone or polyproline are known to exhibit substantially longer half-lives in blood following intravenous injection than do the corresponding unmodified compounds (Abuchowski et al., 1981; Newmark et al., 1982; and Katre et al., 1987). Such modifications may also

increase the compound's solubility in aqueous solution, eliminate aggregation, enhance the physical and chemical stability of the compound, and greatly reduce the immunogenicity and reactivity of the compound. As a
5 result, the desired *in vivo* biological activity may be achieved by the administration of such polymer-compound adducts less frequently or in lower doses than with the unmodified compound.

10 Attachment of polyethylene glycol (PEG) to compounds is particularly useful because PEG has very low toxicity in mammals (Carpenter et al., 1971). For example, a PEG adduct of adenosine deaminase was approved in the United States for use in humans for the treatment of severe
15 combined immunodeficiency syndrome. A second advantage afforded by the conjugation of PEG is that of effectively reducing the immunogenicity and antigenicity of heterologous compounds. For example, a PEG adduct of a human protein might be useful for the treatment of disease
20 in other mammalian species without the risk of triggering a severe immune response. The carrier includes a microencapsulation device so as to reduce or prevent an host immune response against the compound or against cells which may produce the compound. The compound of the
25 present invention may also be delivered microencapsulated in a membrane, such as a liposome.

Polymers such as PEG may be conveniently attached to one or more reactive amino acid residues in a protein such as
30 the alpha-amino group of the amino terminal amino acid, the epsilon amino groups of lysine side chains, the sulfhydryl groups of cysteine side chains, the carboxyl groups of aspartyl and glutamyl side chains, the alpha-carboxyl group of the carboxy-terminal amino acid,
35 tyrosine side chains, or to activated derivatives of glycosyl chains attached to certain asparagine, serine or threonine residues.

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Numerous activated forms of PEG suitable for direct reaction with proteins have been described. Useful PEG reagents for reaction with protein amino groups include active esters of carboxylic acid or carbonate derivatives, particularly those in which the leaving groups are N-hydroxysuccinimide, p-nitrophenol, imidazole or 1-hydroxy-2-nitrobenzene-4-sulfonate. PEG derivatives containing maleimido or haloacetyl groups are useful reagents for the modification of protein free sulfhydryl groups. Likewise, PEG reagents containing amino hydrazine or hydrazide groups are useful for reaction with aldehydes generated by periodate oxidation of carbohydrate groups in proteins.

The pathologic hallmarks of Alzheimer's disease (AD) are intracellular and extracellular deposition of filamentous proteins which closely correlates with eventual neuronal dysfunction and clinical dementia (for reviews see Goedert, 1993; Haas et al., 1994; Kosik, 1994; Trojanowski et al., 1994; Wischik, 1989). Amyloid- β peptide ($A\beta$) is the principal component of extracellular deposits in AD, both in senile/diffuse plaques and in cerebral vasculature. $A\beta$ has been shown to promote neurite outgrowth, generate reactive oxygen intermediates (ROIs), induce cellular oxidant stress, lead to neuronal cytotoxicity, and promote microglial activation (Behl et al., 1994; Davis et al., 1992; Hensley, et al., 1994; Koh, et al., 1990; Koo et al., 1993; Loo et al., 1993; Meda et al., 1995; Pike et al., 1993; Yankner et al., 1990). For $A\beta$ to induce these multiple cellular effects, it is likely that plasma membranes present a binding protein(s) which engages $A\beta$.

A link between cell death, mutant presenilins, and RAGE is proposed. An investigation into whether increased expression of RAGE promotes cellular interactions with $A\beta$, thereby increasing cell stress and, in the presence of mutant presenilin 2, synergistically drives cells into

5

This invention is illustrated by examples set forth in the Experimental Details section which follows. This section is provided to aid in an understanding of the invention but is not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

EXPERIMENTAL DETAILS

5 Example 1: Presenilin-2 Enhances Cytotoxicity Due To Amyloid- β Peptide Interaction With RAGE On Neurons

Introduction

10 Extracellular accumulations of amyloid in neuritic plaques
composed predominately of amyloid-beta peptide ($A\beta$) are
pathognomonic features of Alzheimer's disease (AD) (Haas
et al., 1994; Kosik, et al., 1994; Yankner, et al., 1996;
Goedert, et al., 1993; Trojanowski, et al., 1994). These
15 lesions increase in number and volume over time resulting
in an apparent replacement of the neuronal cell
population (Haas et al., 1994; Kosik, et al., 1994;
Yankner, et al., 1996; Goedert, et al., 1993; Trojanowski,
et al., 1994; Cummings, et al., 1995), and are closely
20 associated with neuronal toxicity leading to dementia.

In AD it is widely accepted that later in the course of
the disease, when $A\beta$ fibrils are abundant, nonspecific
interactions of such fibrils with the cell surface may be
25 frequent and disruptive for cellular functions (Yankner,
et al., 1990; Cotman, et al. 1995; Mattson, et al., 1995;
Hensley, et al., 1994; Behl, et al., 1994; Younkin, et
al., 1995). $A\beta$ fibrils can disrupt plasma membranes,
causing changes in course of the disease, when $A\beta$ fibrils
30 are present at lower levels (and monomers/oligomers
predominate, as opposed to fibrils), higher affinity
interactions with cellular surfaces are more likely to be
relevant. The immunoglobulin superfamily receptor RAGE
(receptor for advanced glycation end product), expressed
35 by neurons and microglia, is present at high levels in AD
brain, both in areas of affected brain parenchyma (at the
antigen and mRNA levels) and in nearby vasculature. RAGE

is a receptor with nanomolar affinity for A β monomer/oligomer, as well as for fibrils (Yan, et al., 1996). In culture, cells expressing RAGE display enhanced susceptibility to A β -induced cellular dysfunction compared with those expressing lower levels of RAGE, or those in which the receptor is blocked. Consistent with a role for A β -receptor interactions in early perturbation of neuronal functions, relevant outcomes of A β binding to neuronal RAGE include activation of nuclear factor-KB (NF-kB), induction of heme oxygenase type 1 and expression of macrophage-colony stimulating factor (M-CSF), each of which can be demonstrated in AD brain (Yan, et al., 1996; Yan, et al. 1997).

Mutant presenilins 1 and 2 are closely associated with early onset familial AD (Haas, et al., 1996; Dwji, et al., 1996; Tanzi, et al., 1996; Hardy, et al. 1997). Furthermore, a relationship between presenilin-2 and cellular pathways eventuating in programmed cell death is indicated; a mutant form of presenilin-2 results in expression of a molecule causing increased basal apoptosis in nerve growth factor-differentiated PC12 cells (Wolozin et al., 1996).

Results

Characterization of Transfected Cells. PC12 cells stably transfected to overexpress RAGE showed increased levels of RAGE, compared with nontransfected controls, by immunostaining (Figs.1A-1B). Furthermore, immunoblotting of cell extracts demonstrated increased RAGE antigen in RAGE tranfectants versus mock-transfected controls (Fig. 2). Transfection of cells with the mutant presenilin 2 construct has been shown to result in overexpression of presenilin-2 antigen (Wolozin et al., 1996).

Induction of apoptosis. For apoptosis studies, PC12

cultures, either mock-transfected (control), stably transfected with the RAGE-bearing construct or stably transfected with the RAGE-bearing construct and transiently transfected with the mutant presenilin-2 bearing construct, and were then exposed to A β (0.3 or 1 μ M) for 24 hrs. At the end of this time, apoptosis was determined using the TUNEL assay by counting positively staining nuclei per twenty high-power fields. Under these conditions, mutant presenilin-2 by itself has little effect on apoptosis, as shown previously (Fig. 3). However, cells co-transfected to express mutant presenilin-2 and RAGE showed a dramatic increase in apoptosis at A β concentrations of both 0.3 and 1 μ M.

Discussion

Alzheimer's disease is likely to result from a combination of factors resulting in increased production of A β , enhanced susceptibility of cells to the effects of A β , and an augmented apoptotic response to environmental stimuli. RAGE tethers A β to the cell surface; this led us to consider the hypothesis that increased RAGE expression, along with elevated levels of mutant presenilin-2, might enhance A β toxicity. As demonstrated herein, this general concept proved to be true. However, the synergistic interaction of these two factors resulted in dramatically increased apoptosis; the latter suggesting a potent mechanism for inducing neuronal death in Alzheimer's disease. The interaction of mutant presenilin-2 with RAGE in transfected cultured cells, as well as in transgenic mice, provides a useful model system for investigating the pathobiology of AD and as a model system for identifying and testing neuroprotective therapeutics.

Experimental Procedures

Generation of Stable Transfected Cells. PC12 cells, grown

as described and transfected with pcDNA3-RAGE (the latter comprised of the human RAGE cDNA) (Yan, et al., 1996) using lipofectamine (12 μ g/ml) according to the manufacturer's instructions (Gibco-BRL). Cells were
5 maintained in the presence of G418. Transient transfection was performed with a vector including mutant presenilin-2 (N141 mutant) (Wolozin et al., 1996), using lipofectamine, as described (Wolozin et al., 1996). Immunoblotting and immunostaining of PC12 cells for RAGE
10 was performed as described previously (Yan, et al., 1996).

Apoptosis assay. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed as described using a kit from Travigen, a
15 peroxide-based TACS-TdTkit (Woolozin, et al., 1996). A β (comprised of residues 1-42) was synthesized, HPLC purified, and purchased from a commercial supplier.

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What is claimed is:

What is claimed is:

- 5 1. A method for evaluating the ability of a compound to inhibit neurotoxicity which comprises:
 - 10 (a) contacting a cell which expresses a receptor for advanced glycation end product protein and a mutant presenilin-2 protein in a cell culture and the compound;
 - (b) determining the level of cell death in the cell culture; and
 - 15 (c) comparing the level of cell death determined in step (b) with the amount determined in the absence of the compound so as to evaluate the ability of the compound to inhibit neurotoxicity.
- 20 2. The method of claim 1, wherein the cell is a neuronal cell, a glial cell, a microglial cell, an astrocyte, an endothelial cell, a mononuclear cell, a tumor cell, or a PC12 cell.
- 25 3. The method of claim 1, wherein the compound is a peptide, a peptidomimetic, a nucleic acid, a polymer, or a small molecule.
4. The method of claim 1, wherein the compound is bound to a solid support.
- 30 5. The method of claim 1, wherein the mutant presenilin-2 is overexpressed.
- 35 6. A method for evaluating the ability of a compound to inhibit binding of an amyloid- β peptide to a receptor for advanced glycation end product which comprises:
 - (a) contacting a cell which expresses a mutant

presenilin-2 protein and a receptor for advanced glycation end product protein with amyloid- β protein and the compound;

5 (b) determining the amount of amyloid- β peptide bound to the cell;

(c) comparing the amount of bound amyloid- β peptide determined in step (b) with the amount determined in the absence of the compound so as to evaluate the ability of the compound to inhibit binding of the amyloid- β peptide to the receptor for advanced glycation end product.

7. The method of claim 1, wherein the cell is a neuronal cell, a glial cell, a microglial cell, an astrocyte, an endothelial cell, a mononuclear cell, a tumor cell, or a PC12 cell.

8. The method of claim 1, wherein the compound is a peptide, a peptidomimetic, a nucleic acid, a polymer, or a small molecule.

9. The method of claim 1, wherein the compound is bound to a solid support.

10. The method of claim 1, wherein the mutant presenilin-2 is overexpressed.

11. A pharmaceutical composition which comprises a compound capable of inhibiting neurotoxicity identified by the method of claim 1, and a pharmaceutically acceptable carrier.

12. The pharmaceutical composition of claim 11, wherein the carrier is a diluent, an aerosol, a topical carrier, an aqueous solution, a nonaqueous solution or a solid carrier.

13. A method for treating a neurodegenerative condition in a subject which comprises administering to the subject an amount of the pharmaceutical composition of claim 11, effective to treat the neurodegenerative condition in the subject.
14. The method of claim 13, wherein the neurodegenerative condition is associated with Alzheimer's disease, diabetes, senility, renal failure, hyperlipidemic atherosclerosis, neuronal cytotoxicity, Down's syndrome, dementia associated with head trauma, amyotrophic lateral sclerosis, myasthenia gravis, multiple sclerosis or neuronal degeneration.
15. The method of claim 13, wherein the neurodegenerative condition is associated with degeneration of a neuronal cell in the subject.
16. The method of claim 13, wherein the neurodegenerative condition is associated with the formation of an amyloid- β peptide fibril.
17. The method of claim 13, wherein the neurodegenerative condition is associated with aggregation of amyloid- β peptide.
18. The method of claim 13, wherein the neurodegenerative condition is associated with infiltration of a microglial cell into a senile plaque.
19. The method of claim 13, wherein the neurodegenerative condition is associated with activation of a microglial cell by an amyloid- β peptide.
20. The method of claim 13, wherein the subject is a human.

21. A transgenic non-human animal whose somatic and germ cells contain and overexpress a gene encoding human presenilin-2 protein and whose somatic and germ cells contain and overexpress a gene encoding human receptor for advanced glycation end product protein, the genes having been introduced into the animal or an ancestor of the animal at an embryonic stage and wherein the gene may be operably linked to an inducible promoter element.
22. The animal of claim 21, wherein the animal is a mouse.
23. The animal of claim 21, wherein the gene encoding human presenilin-2 protein is a mutant gene.
24. A method for identifying whether a compound is capable of ameliorating a neurodegenerative condition in an animal comprising:
- (a) administering the compound to the transgenic animal of claim 10, wherein the animal exhibits a neurodegenerative condition;
 - (b) measuring the level of neurodegeneration in the animal; and
 - (c) comparing the level of neurodegeneration measured in step (b) with the level of neurodegeneration measured in the animal in the absence of the compound so as to identify whether the compound is capable of ameliorating the neurodegenerative condition in the animal.
25. The method of claim 24, wherein the neurodegenerative condition is associated with Alzheimer's disease, diabetes, senility, renal failure, hyperlipidemic atherosclerosis, neuronal cytotoxicity, Down's syndrome, dementia associated with head trauma, amyotrophic lateral sclerosis, myasthenia gravis,

multiple sclerosis or neuronal degeneration.

26. The method of claim 24, wherein the neurodegenerative condition is associated with degeneration of a neuronal cell in the subject.
27. The method of claim 24, wherein the neurodegenerative condition is associated with the formation of an amyloid- β peptide fibril.
28. The method of claim 24, wherein the neurodegenerative condition is associated with aggregation of amyloid- β peptide.
29. The method of claim 24, wherein the neurodegenerative condition is associated with infiltration of a microglial cell into a senile plaque.
30. The method of claim 24, wherein the neurodegenerative condition is associated with activation of a microglial cell by an amyloid- β peptide.
31. A cell comprising a recombinant nucleic acid which comprises DNA encoding mutant presenilin-2 protein and encoding receptor for advanced glycation end product protein.
32. The cell of claim 31 wherein the cell secretes mutant presenilin-2 and RAGE is transmembrane.
33. The cell of claim 31 wherein the cell is a neuronal cell, an endothelial cell, a glial cell, a microglial cell, an astrocyte, a smooth muscle cell, a somatic cell, a bone marrow cell, a liver cell, an intestinal cell, a germ cell, a myocyte, a mononuclear phagocyte, an endothelial cell, a tumor cell, a stem cell, or a PC12 cell.

A Method For Evaluating The Ability Of A Compound To
Inhibit Neurotoxicity

5 Abstract of the Disclosure

This invention provides a method for evaluating the ability of a compound to inhibit neurotoxicity which comprises (a) contacting a cell which expresses a receptor
10 for advanced glycation end product protein and a mutant presenilin-2 protein in a cell culture and the compound; (b) determining the level of cell death in the cell culture; and (c) comparing the level of cell death determined in step (b) with the amount determined in the
15 absence of the compound so as to evaluate the ability of the compound to inhibit neurotoxicity.

The invention also provides a method for evaluating the ability of a compound to inhibit binding of an amyloid- β
20 peptide to a receptor for advanced glycation end product which comprises (a) contacting a cell which expresses a mutant presenilin-2 protein and a receptor for advanced glycation end product protein with amyloid- β protein and the compound; (b) determining the amount of amyloid- β
25 peptide bound to the cell; (c) comparing the amount of bound amyloid- β peptide determined in step (b) with the amount determined in the absence of the compound so as to evaluate the ability of the compound to inhibit binding of the amyloid- β peptide to the receptor for advanced
30 glycation end product.

The invention also provides a transgenic non-human animal whose somatic and germ cells express mutant human presenilin-2 protein and human receptor for advanced
35 glycation end product protein.

FIG. 1A

Mock-transfected



FIG. 1B

RAGE-transfected

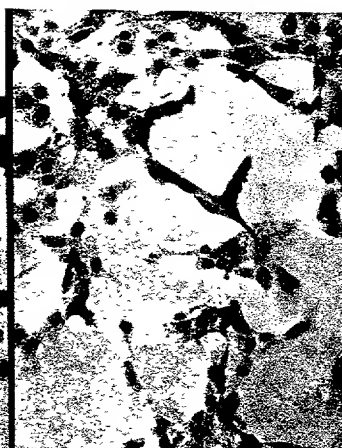


FIG. 2

